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The University of Southern Mississippi

Species Delimitation and Phylogenetic Relationships of the Wild Yams (*Dioscorea*)
Native to Eastern North America

by

Tori A. Collins

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Biological Sciences

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Abstract

Most taxonomic treatments currently recognize two to three species of native yams in eastern North America: *Dioscorea villosa*, *D. floridana*, and sometimes *D. quaternata*, a segregate of *D. villosa*. Earlier authors (e.g., J. K. Small) had recognized as many as five species (with *D. hirticaulis* and *D. glauca* also as segregates of *D. villosa*). Key morphological features in distinguishing these putative species are rhizome morphology (long and cord-like vs. thick and contorted), number of first leaves (1–3 vs. 4–7), and habitat (sandy, rocky, swampy). Unfortunately, these critical features are rarely collected and preserved on herbarium sheets, given the length and twining nature of these perennial vines. Instead, herbarium material often consists of the terminal part of the vine, usually less than 0.5 m, and reproductive parts of a single sex. To assess species boundaries, then, representatives of the putative species were collected and assessed for genetic variation. Unique haplotypes corresponding to the morphological units would support the hypothesis of separate species; common haplotypes would be ambiguous, that is, would neither support nor refute the hypothesis of separate species, but would provide baseline data for future studies. *Dioscorea floridana* and a broadly circumscribed *D. villosa* were recovered as genetically distinct, but no variation was found in the *D. villosa* complex. Given these data, boundaries within the *D. villosa* complex remain ambiguous. Combined with data from other studies, these DNA data were then used to infer relationships of the native U.S. species to other species in the genus as well to the species in *Dioscorea* section *Stenophora*.

Acknowledgments

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CHAPTER I

STATEMENT OF THE PROBLEM

Dioscorea is a genus of flowering plants with engorged tubers, which are often called “yams.” Some species have edible tubers (Chair et al. 2011), and some are a source of steroid hormones used to combat menopause and act as a contraceptive (Correll et al. 1955; Applezweig 1962). Many scientists in North America currently recognize two native species, following the treatment in *Flora of North America* (Raz 2002), but this number of species is contested in the literature. Others claim as many as five species (e.g., Deam 1940; Small 1933). Morphological data have not resolved the species problem, especially because the long, unisexual vines are commonly collected without the tuber and only one sex is available on an individual. Thus, the available material in museums is often inadequate for comparison. A genetic study was undertaken to more precisely test the boundaries of the native species of wild yams. Putative “species” were tested to determine if they have unique DNA haplotypes (sequences), which would indicate the lack of gene flow. By combining the morphology of yams with genetic data, this study attempts to establish a better understanding of the diversity of native species of wild yams and to permit more precise studies of the medical components of the *Dioscorea* native to eastern North America.

CHAPTER II

REVIEW OF THE LITERATURE

Species delimitation is the process of identifying biodiversity at the species-level (Carstens 2013; de Queiroz 2007). Species are the foundational units of living things. Within biology, however, there is a lot of controversy about what “species” *theoretically* are (Mayden 1997) and then how to recognize them *practically* (de Queiroz 2007). While the biological species concept (“BSC,” Mayr 1942)—where species are defined as individuals with the capacity to interbreed—has been utilized to define many species, plant species are usually not defined by this model because many groups of plants readily hybridize. Many plants are capable of producing viable offspring with a host of similar or related entities. If plant species were recognized using a biological species concept, the current “species” would have to be broadened, ignoring logical division based on morphology, ecology, genetics, and evolution (Templeton 1992). Historically, plant species have been recognized predominantly with morphological data, but this method can result in perceived similarities with no underlying genetic similarity.

The phylogenetic species concept (Nixon & Wheeler 1990), which is more commonly used in plants, focuses on diagnostic features that are constantly different between populations. The goal is to find the smallest aggregation of a lineage based on character states. These character states are irreversible transformations that mark the point at which a new species has formed (Nixon & Wheeler 1990). These differences can be caused by changes in appearance, in structure, in the geographic habitats, or in the

genetic information. However, some differences could simply be variation of a trait within the same species. The difference between a trait (difference *within* a species) and a character state (difference *between* species) must be studied carefully and with robust sampling. Nixon and Wheeler state “if species are delimited too broadly, some opportunities for cladistic resolution are lost. If delimited too narrowly, the results become spurious because they rest on polymorphic traits within populations rather than upon phylogenetically informative characters” (1990: 213). It is important to differentiate variation among species from variation within a species. This distinction is difficult to identify, depending on the data available, and as a result, some populations are divided differently based on different interpretations of the available data.

Dioscorea is a genus commonly known as yams. North America has six commonly accepted species according to the *Flora of North America* (2002), four of which are introduced species. The two native species are *Dioscorea floridana* and *D. villosa*, which are grouped into the section *Macropoda* due to the counter-clockwise twining of the stems, sepals/petals united at the base, staminate (male) flowers with six stamens, and broad capsules (Al-Shebaz & Schubert 1989; Raz 2002). Some authors have united the section *Macropoda* with the section *Eustenophora* to form the section *Stenophora* (Burkill 1960; Wilkin et al. 2005). Understanding relationships among both sections has been seen as critical in understanding the genus (Wilkin et al. 2005).

Dioscorea floridana occurs predominantly in the state of Florida. This species has yellow rhizomes, unbranched stems, articulated nodes, and flowers in the late spring to mid-summer. *Dioscorea floridana* inhabits the edges of swamps, moist to dry pine

forests, and sandy soils. *Dioscorea villosa* is more variable. The rhizome is brown. The stems can be unbranched or highly branched, and most are narrowly winged. Variation can also be seen in the length between internodes. The rhizome can take one of two distinct shapes, either thin and linear or thick and irregularly contorted. *Dioscorea villosa* flowers in mid-spring to summer. Habitats include bogs, swamps, marshes, margins of freshwater bodies, creek bottoms, rocky or sandy soils, moist to dry woods, and limestone or talus slopes (Raz 2002).

Due to the highly polymorphic nature of this latter species, many systematists have proposed a division of the *D. villosa* species complex into a group of true *D. villosa*, as well as *D. quaternata*, *D. glauca*, and *D. hirticaulis*. It has been proposed that true *D. villosa* has aerial stems that can grow to be up to five meters long, thin and linear rhizomes, alternating leaves, and is predominately found in moist woods or thickets (e.g., Correll & Johnston 1979; Gleason & Cronquist 1991; Small 1933; Yatskievych 1999). The leaves are generally alternating, though they occasionally occur in clusters of three, especially at the first node (Yatskievych 1999). *Dioscorea quaternata* has aerial stems which can only grow to be up to three meters long, the rhizomes are thick and contorted, and can be found in thickets, rocky slopes, banks, moist hemlocks, or woods (e.g., Clewell 1985; Correll & Johnston 1979; Gleason & Cronquist 1991; Small 1933; Yatskievych 1999). The leaves are in sets of three or four below and then alternate above (Yatskievych 1999). *Dioscorea glauca* is described much like *D. quaternata*, but is said to be bigger and the leaves are described as more glaucous or waxy (Small 1933). *Dioscorea hirticaulis* has slender lateral branches, produces few flowers or fruits, and is

commonly found in swamps (Small 1933). *Dioscorea hirticaulis* is the only one of the four species that is pale pubescent underneath (Small 1933). Below is a dichotomous key highlighting the distinguishing features among the five putative species.

A dichotomous key of the described species of native *Dioscorea*

- 1. Rhizome yellow and cordlike; staminate panicles clustered in leaf axils.....*D. floridana*
- 1. Rhizome brown, sometimes cordlike and sometimes twisted, contorted, or thick; staminate panicles solitary in leaf axils
 - 2. Lower leaves alternating singly or occasionally clustered in sets of three leaves at the first node
 - 3. Pistillate raceme with many flowers, many fruits at maturity; stem internodes glabrous*D. villosa*
 - 3. Pistillate raceme with few flowers, one to four fruits at maturity; stem internodes pubescent *D. hirticaulis*
 - 2. Lower leaves clustered in sets of four to seven leaves
 - 4. Leaf blade green below; sepal length less than or equal to 1.5 mm
.....*D. quaternata*
 - 4. Leaf blade grayish waxy below; sepal length greater than 1.5 mm.....
.....*D. glauca*

As alluded to earlier, not all scientists agree on which species to recognize. Some argue that *D. floridana* and *D. villosa* are the only native species of wild yams (e.g.,

Britton & Brown 1970; Raz 2002; Weakley 2012). Others recognize *D. quaternata* (e.g., Al-Shebaz & Schubert 1989; Clewell 1985; Gleason & Cronquist 1991) as well, and a few older treatments recognize five species (e.g., Deam 1940; Small 1933). Field-collected and herbarium samples used in this study were identified using the above key.

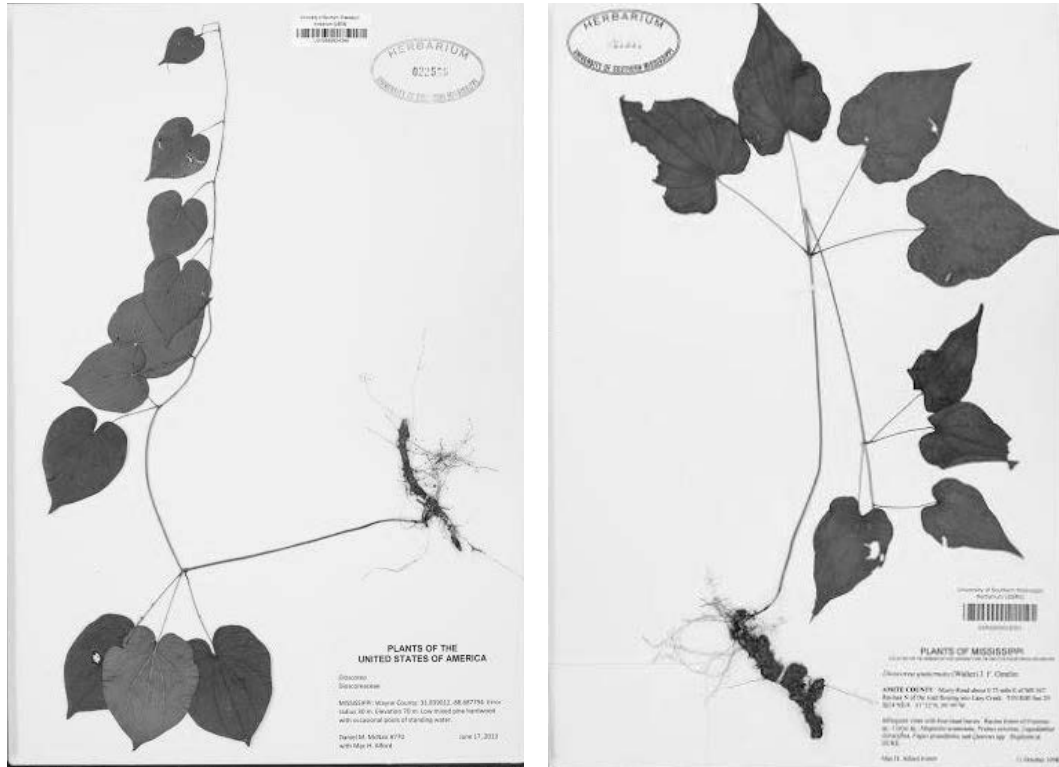


Figure 1. Herbarium specimens of the *D. villosa* species complex. The left sample represents *D. villosa* sensu stricto, while the right sample represents *D. quaternata*.

As seen in the image above, *D. villosa* has three leaves at first node. The thin and linear rhizome is also most commonly seen in specimens identified as *D. villosa*.

Dioscorea quaternata can be recognized based on the presence of four or more leaves at the first node. The rhizome is also an example of the thick and contorted shape more

commonly seen in *D. quaternata*. Below is a table of various systematists and the species which they recognize in their respective regions of study (Table 1).

Al-Shehbaz and Schubert (1989) compiled data on *Dioscorea* which describe each of the five species that could be present in North America. The data they compiled describe distinguishing features of all five species, although they do not recognize all five species. They state that *D. hirticaulis* is probably just a subspecies of *D. villosa* (subspecies meaning that they can interbreed but are geographically mostly separate) and that *D. glauca* is just a subspecies of *D. quaternata* (1989). Small (1933) and Deam (1940) recognized all five species. Others take the middle road, such as Yatskievych (1999), who recognized both *D. villosa* and *D. quaternata*, but stated that the two are hard to distinguish aside from the rhizome shapes and lower leaf twisting patterns. Some do not believe these morphological features are enough to divide the species complex, such as Britton and Brown (1970), Raz (2002), and Weakley (2012). Most of the treatments that recognize only *D. villosa* state that the species (or species complex) likely needs to be further studied. Al-Shehbaz and Schubert describe the areas in which *D. floridana*, *D. villosa*, *D. quaternata*, *D. glauca*, and *D. hirticaulis* have been identified (1989). These data were compiled with other regional treatments (Raz 2002; Small 1933) to create the map below (Figures 2–3).

Table 1. Species of *Dioscorea* recognized in treatments of the genus. Gray boxes indicate that the putative species is not known from that particular region and is not applicable.

		<i>D. villosa</i>	<i>D. hirticaulis</i>	<i>D. quaternata</i>	<i>D. glauca</i>	<i>D. floridana</i>
Al-Shehbaz & Schubert (1989)	Dioscoreaceae in the Southeastern US	X		X		X
Raz (2002)	Flora of North America	X				X
Small (1933)	Southeastern Flora	X	X	X	X	X
Gleason (1991)	Vascular Plants of the NE US and Adjacent Canada	X		X		
Britton & Brown (1970)	Flora of the Northern US and Canada	X				
Weakley (2012)	Flora of the Southern and Mid-Atlantic States	X				X
Clewell (1985)	Vascular Plants of the Florida Panhandle			X		X
Ward (1977)	Keys to the Flora of Florida			X		X
Yatskievych (1999)	Flora of Missouri	X		X		
Correll & Johnston (1979)	Vascular Plants of Texas	X		X		
Diggs et al. (2006)	Flora of East Texas	X		X		
Wofford (1989)	Vascular Plants of the Blue Ridge	X				
Jones (2005)	Plant Life of Kentucky	X				
Deam (1940)	Flora of Indiana	X	X	X	X	
Jones (1971)	Flora of Illinois	X		X		
Voss (1972)	Michigan Flora	X				
Braun (1967)	Monocots of Ohio	X		X		
Strausbaugh & Core (1978)	Flora of West Virginia	X		X		
Rhoads & Block (2000)	Plants of Pennsylvania	X		X		
Eilers & Roosa (1994)	Vascular Plants of Iowa	X				
Rolfsmier & Steinauer (1999)	New Floristic Record for Nebraska			X		

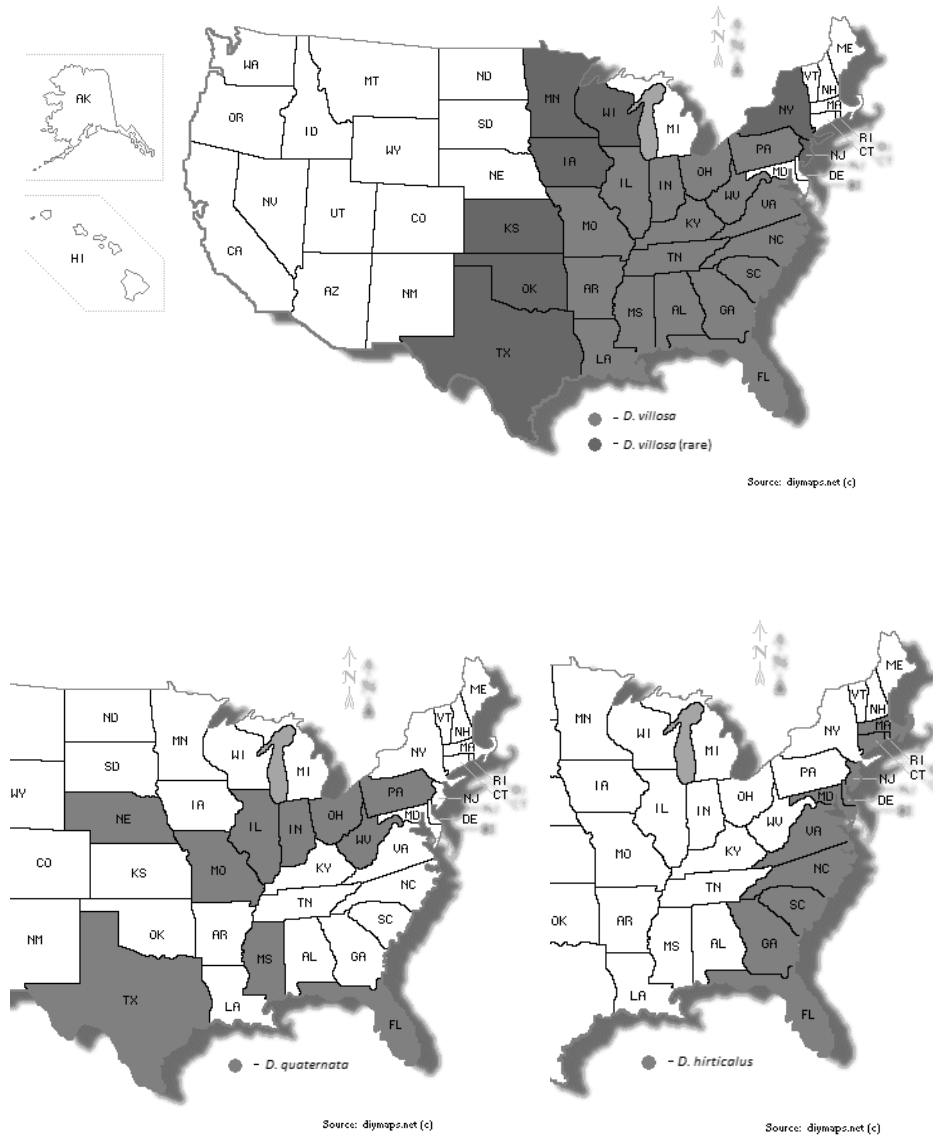


Figure 2. Distribution of *Dioscorea villosa* sensu stricto, *D. quaternata*, and *D. hirticaulis*, based on national and regional treatments of the genus.

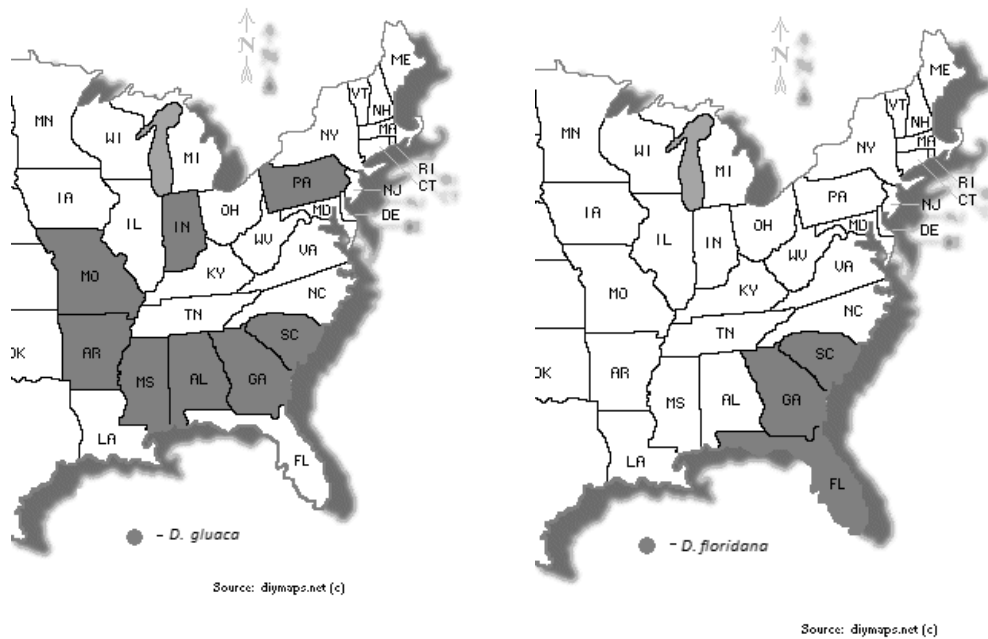


Figure 3. Distribution of *Dioscorea glauca* and *D. floridana*, based on national and regional treatments of the genus.

The above maps show the distribution of the putative species as inferred from the literature listed in Table 1 (Al-Shehbaz & Schubert 1989; Raz 2002). *Dioscorea villosa* and *D. quaternata* are fairly widespread. Since the two species are currently recognized as one species in *Flora of North America*, their precise distributions are not fully known, so the maps represent estimated ranges. Al-Shehbaz and Schubert (1989) state that most of the specimens that are currently called *D. villosa* may actually be *D. quaternata*. They explain by saying that *D. villosa* is actually found only in the states of the coastal plain (Al-Shehbaz & Schubert 1989). The maps also show that *D. hirticaulis* has been predominantly recognized along the eastern sea-board (Al-Shehbaz & Schubert 1989).

Dioscorea glauca has been described in Missouri, Pennsylvania, Indiana, and from South Carolina to Arkansas, but is rarely found in the coastal plain (Deam 1940; Small 1933).

Clearly, more data—in addition to morphology—are needed to resolve the species problem in *Dioscorea*. Genetic sequences can provide greater insight because sufficient genetic data can help us distinguish between features that define a species and features that are just variation within a species (Olmstead & Palmer 1994). In other words, if the morphological patterns of the putative species perfectly match the genetic patterns, the putative species are probably “real.” If they do not match, it indicates that there is gene flow (interbreeding) among the putative units and that they do not have a divergent history. This is especially the case if we are able to collect more than one putative species at one locality. If they are distinct genetically in one locality, they are probably “good” species. Otherwise, given their proximity, we would expect them to interbreed or be closely related and share some features. If variation is found, a population genetics tool called “isolation by distance” could be used to assess whether the association between the genetic similarity or differences of a two populations and geographic distance between those populations is statistically significant using the Mantel test, which would show whether the change in terrain between the putative species is biologically relevant (Bohonak 2002).

However, to study genetic variation, suitable genes or other genetic regions must first be selected. The region needs to be present in every individual and easy to copy, amplify, and sequence. The selected gene region also needs to be variable enough, meaning it is likely to be different from species to species or even among populations. It

also needs to be variable in the sense that it has changed enough among putative species so that a pattern can be observed.

In plants, some non-coding regions of the plastid DNA fit these criteria of variation such as *matK*, *psbA-trnH*, *rrn4-5-trnN*, *ccsA-ndhD*, and *ycf6-psbM* (Ipek et al. 2014; Johnson & Soltis 1995; Scarcelli et al. 2011; Shaw et al. 2005; Storchova & Olson 2007; Sun et al. 2012). Regions such as *psbA-trnH* are highly polymorphic in many angiosperms, making them a good starting point (Storchova & Olson 2007). Some nuclear DNA regions, such as the *Pgi* locus and the internal transcribed spacer (ITS), also fit the parameters (Ipek et al. 2014; Terauchi et al. 1997). The plastid regions *matK* and *psbA-trnH* have shown success in delimiting native Chinese species of *Dioscorea* (Sun et al. 2012). The regions *rrn4-5-trnN*, *ccsA-ndhD*, and *ycf6-psbM* have been recommended for use in phylogenetic studies of monocotyledon plant groups (Scarcelli et al. 2011; Shaw et al. 2005); Dioscoreaceae are monocots. A past study of another wild yam, *Dioscorea tokoro*, noted that the *Pgi* gene region can be highly polymorphic with a large variety of primers allowing for specific study parameters to be set (Terauchi et al. 1997). A successful species delimitation of *Dioscorea* using DNA sequences has not been published using ITS, although it could still be effective in this study since it has been effective in closely related species (Ipek et al. 2014). Based on this understanding of sequences for delimitation in wild yams, sequences of these nuclear and plastid regions were tested and compared to determine if differences between the putative species of yams exist. If the putative species show variation within more than one gene, it gives strong indication that the differences represent character states, meaning that the

differences have accumulated over time without gene flow. The collected genetic information would then be correlated to morphological features to determine which putative species are “real.” This will not only determine if more than one species exist but which of the five species exists. It is hypothesized that the genetic features will match the morphological features, supporting a division of the species complex.

CHAPTER III

METHODOLOGY

A total of 17 samples of three of the five putative species of *Dioscorea* and a close relative (*D. floridana*) were collected from various regions of eastern North America. The sample collection included specimens from the putative species *D. villosa* and *D. quaternata*, paying special attention to the presence or absence of each of the morphological forms at each locality. Among these 17 samples, 7 were identified as “true” (typical, or sensu stricto) *D. villosa*, while 9 were identified as *D. quaternata*. The different morphological forms were found in close proximity to one another when sites were searched. Identification was primarily based on the appearance of the rhizome. The two remaining samples did not contain a rhizome for confident identification. Only one sample was obtained of the closely related *D. floridana*. Samples were obtained from the field, from preserved specimens in approved herbaria, or collected by botanists in regions that could not be collected from directly. Most samples were collected directly from the field. Samples collected in the field were dried, numbered, and mounted in the typical fashion. The preserved samples were added to the herbarium of the University of Southern Mississippi (USMS) after a fragment was removed for DNA extraction. Samples were also collected from the existing specimens in the university’s herbarium. A sample of *D. floridana* was obtained from a colleague at the University of Florida. An inclusive list of the samples is provided.

Table 2: Samples collected, with identifications based on morphological features. The repeat voucher consisted of multiple individual plants.

Putative Species	Voucher	Extraction Sample #
<i>D. villosa</i>	McNair 682	306
<i>D. villosa</i>	Alford 4365	307
<i>D. quaternata</i>	Alford et al. 4374	308
<i>D. villosa</i> (?)	Howell 90	321
<i>D. villosa</i> (?)	MacDonald 9479	322
<i>D. quaternata</i>	MacDonald 12874	323
<i>D. quaternata</i>	Alford 1669	324
<i>D. quaternata</i>	Alford 951	325
<i>D. floridana</i>	Majure 4467	326
<i>D. villosa</i>	McNair 1927	331
<i>D. villosa</i>	McNair 1927	332
<i>D. villosa</i>	McNair 1927	333
<i>D. villosa</i>	McNair 1927	334
<i>D. villosa</i>	McNair 1927	335
<i>D. quaternata</i>	McNair 1919	337
<i>D. quaternata</i>	McNair 1919	338
<i>D. quaternata</i>	McNair 1919	339
<i>D. quaternata</i>	McNair 1919	340

Once the samples were obtained, a small sample of the leaf tissue was removed for testing. Genetic material was extracted from each of the samples using a DNeasy Plant Mini Kit from Qiagen (Valencia, CA). The first seven steps in the instruction manual were omitted from the extraction process. Instead, a small amount of the plant tissue—a piece of the fragment obtained earlier—were liquefied by grinding the tissue in 500 μ L of Buffer AP1 using a mortar and pestle. The sample was ground until no visible fragments of plant tissue remained. The mixture was then transferred to pre-labeled 1.5 μ L capped tubes. Once transferred, the tube was placed on a heating element and agitated over a period of 10 minutes. The extraction then followed the recommended steps from eight all the way to the end. Optional steps were retained. The resulting DNA was also suspended in buffer—as opposed to water—to maximize the sample's time of usability. The extracted DNA was given a number that corresponds with the sample from which it was drawn. These numbers were included in Table 2 as the sample number.

Samples were then amplified through the polymerase chain reaction (PCR) to produce multiple gene copies of the sequences to be studied. Copies were made of a total of 11 DNA regions utilizing a total of 22 primers. The primers and the corresponding DNA sequences utilized for this study can be seen in the table below.

Table 3: Regions of nuclear and plastid DNA selected for amplification and phylogenetic inference.

Primer	Primer Sequences Utilized	
<i>trnH-psbA</i>	Forward	5'-CGCGCATGGTGGATTCAACAATCC-3'
	Reverse	5'-GTTATGCATGAACGTAATGCTC-3'
ITS5-ITS4	Forward	5'-GGAAGTAAAAGTCGTAACAAGG-3'
	Reverse	5'-TCCTCCGCTTATTGATATGC-3'
ETS	Forward	5'-CGCATCGTTCGGTGCATTCTGGG-3'
	Reverse	5'-ACTTACACATGCATGGGTTAATCT-3'
<i>matk</i> _390– <i>matk</i> _1326	Forward	5'-CGATCTATTCATTCAATATTTC-3'
	Reverse	5'-TCTAGCACACGAAAGTCGAAGT-3'
<i>matk</i> _1412– <i>matk</i> _1176	Forward	5'-ATATAATTCTTATGTATGTG-3'
	Reverse	5'-CAATTCATTCAATATTTTCCTT-3'
<i>rrn4-5-trnN</i>	Forward	5'-GYCAAGTGGAAGTGCAGTGA-3'
	Reverse	5'-GGTAGAGCGGTYGGCTGTTA-3'
<i>ccsA-ndhD</i>	Forward	5'-GCAGTRTGGGCTAATGAGG-3'
	Reverse	5'-GGAATGAGYGGTTTTGTTGC-3'
<i>pgi</i> 95.1– <i>pgi</i> 95.5	Forward	5'-AACTTGCTGAGGTGGCTTG-3'
	Reverse	5'-AATGGAGTGGAATGGAAAT-3'
<i>ycf6-psbM</i>	Forward	5'-ATGGATATAGTAAGTCTYGCTTGGGC-3'
	Reverse	5'-ATGGAAGTAAATATTCTYGCATTTATTGCT-3'
<i>rpL32-ndhF</i>	Forward	5'-CCAATATCCCTTYTTCCTTCCAA-3'
	Reverse	5'-GAAAGGTATKATCCAYGMATATT-3'
<i>trnL</i> (c,f)	Forward	5'-CGAAATCGGTAGACGCTACG-3'
	Reverse	5'-ATTTGAACTGGTGACACGAG-3'

The process of PCR utilized a *Taq* polymerase mixture (TaKaRa Ex Taq, TaKara Bio USA, Madison, WI). DNA amplification was enhanced using a PCR additive reagent (TBT-PAR) prepared in the lab in an attempt to counteract the difficulty of DNA amplification in plant materials (Samarakoon et al. 2013). First, 0.5 µL tubes were labeled with the sample number being amplified. The appropriate materials were removed from storage and moved to a container of ice. When the materials were frozen, they were allowed to thaw slowly. Once completely thawed, 8 µL of distilled water was mixed with 10 µL of the TBT-PAR additive reagent in each of the labeled tubes. A total

of 25 μL of the *Taq* mixture was then added to each tube, taking extra precaution to avoid contamination. Then, 2.5 μL of the forward and the reverse primers of the DNA region of interest were injected into the tubes. Finally, the corresponding DNA sample was added to each solution. This process of adding reagents was completed over ice. The tubes were then placed in a Thermo PCR Sprint thermal cycler. The cycler was pre-set so the lid of the container was heated by the time the mixing of the solution had been completed. Samples were then placed around the internal thermostat. The thermal cycler is also pre-programmed to complete the recommended stages for the *Taq* mixture which include three steps: denaturing, annealing, and extension. When the process first begins the samples were held at a temperature of 94° C for three minutes to allow the DNA helices to uncoil and separate. Then the first denaturing occurred at 94° C for 30 seconds, followed by annealing at 60° C for 30 seconds, and completed with extension at 72° C for one minute. This three step process was repeated for 30 cycles by the thermal cycler. Once the 30 cycles had been completed the temperature was held at 4° C until they could be placed in the refrigerator for storage or cleaned.

A portion of the resulting amplified DNA was run out by gel electrophoresis to confirm the amplification of the genetic region of interest. The gel is prepared by mixing 1.5 g of agarose with 150 mL of 1 \times Tris-boric acid-EDTA (TBE) buffer and heating. Upon cooling, the solution becomes a gel. When used, the solution or pre-prepared gel was heated to bring the solid back into a liquid phase. The solution was microwaved for about 30–60 s until the solution was boiling. Once boiling, roughly 30 mL of solution was poured into a 40 mL beaker. The solution was allowed to cool until cool to the touch.

The mixture was then poured into a gel block wedged into a plastic frame to ensure a closed pouring platform. A 10-well comb was placed in the gel block immediately after pouring. The gel sat at room temperature for 15 minutes to solidify. The comb was then removed after the gel solidified. The solidified gel was then placed in an electrophoresis chamber filled with TBE buffer. A small piece of parafilm was labeled according to the DNA being run out. One μL of loading buffer (Qiagen GelPilot Loading Dye, Qiagen, Valencia, CA) was then added above each of the labels. Four μL of the corresponding DNA was added to each of the beads of loading buffer. Four μL of a DNA ladder (Promega Bench Top PCR Markers, Madison, WI) was added to the first well in the gel. The ladder acted as a standard of comparison for the size of the fragments being run out. The proceeding wells were then filled one by one with the dye and DNA mixture in a defined order for future reference. The gel was then run in a Fisher Scientific FB300 at 100 V for 20 minutes. The electrical current caused the DNA to move through the gel over a certain distance depending of the length of the amplified product. This acted as a checking point for contamination. If the resulting DNA had a larger or smaller size than expected, it was likely not the region of interest that had been amplified. The resulting gel run was then soaked in a mixture of 5 μL of ethidium bromide and enough TBE buffer to cover the top of the gel in the soaking chamber that was lined with aluminum foil. The gel was soaked for 20–30 minutes, after which it was placed on an ultraviolet radiating light that caused the dye to fluoresce. A positive result was defined by a glowing band in the expected ladder (size) region. DNA resulting in a positive result was then cleaned

according to the directions outlined in the Qiaquick PCR Purification Kit (Qiagen, Valencia, CA).

Purified samples were then sent off to be sequenced at Eurofins MWG/Operon in Louisville, KY, using the standard procedure of ABI 3730xi DNA sequencers. Completed sequences were returned via e-mail in .ab1 format. The returned sequences were cleaned up by confirming accuracy of the computer analysis and by determining results for ambiguous nucleotide readings using Sequencher version 5.0 (GeneCodes, 2006, Ann Arbor, Michigan). The results of the forward and reverse strands were then combined into a “contig,” utilizing the program’s “Assemble Automatically” function. Cleaned sequences were then exported into ClustalX 2.0.7 (Thompson et al. 1997), which aligns the sequences from all the sequenced DNA samples and which were saved in .gde format and reopened in Winclada 1.00.08 (Nixon, 2002) to compare the base-pairs, find variable sites, and infer phylogenetic trees from the resulting variation. An outgroup DNA sequence was downloaded from GenBank and used as a root—a species not in the complex. The resulting tree determined whether the changes corresponded to morphologically putative species, were correlated to geography, or if no changes existed.

CHAPTER IV

RESULTS

DNA extractions were obtained for all of the samples. A representative group was used for a preliminary round of PCR to test the selected regions for variability. This set included Samples 306, 307, 308, and 326. When results were obtained, more samples were amplified. When the results of these amplifications were run out on a gel, nine primer regions produced banding patterns. Of those nine, five produced clean sequences: *trnH-psbA*, *matK_390-matK_1326*, *rrn4-5-trnN*, *ccsA-ndhD*, and *rpL32-ndhF*. The five resulting clean sequences were then compared for variation. When the clean sequences from *trnH-psbA*, *matK_390-matK_1326*, *rrn4-5-trnN*, and *ccsA-ndhD* DNA regions were aligned, variation was seen between *D. floridana* and the *D. villosa* species complex, but no variation was observed among the putative species of the complex. Since no variation was observed, more samples were not amplified. Upon alignment, the sequences of the *rpL32-ndhF* DNA region showed multiple single nucleotide polymorphisms; however, the variation was *among* the samples representing each putative species. For examples, at aligned position 243, samples 338 (*D. quaternata*) and 306 (*D. villosa*) (direct sequence position 243 and position 223, respectively) show a “G,” while samples 307 (*D. villosa*) and 308 (*D. quaternata*) (direct base position 188 and 187, respectively) show an “A” at the same point. Multiple instances of this can be seen, providing circumstantial evidence that *D. villosa* and *D. quaternata* are part of the same species. Alternatively, this result could be seen as a case of retained ancestral polymorphism.

The obtained sequences were then combined with closely related sequences downloaded from GenBank (www.ncbi.nlm.nih.gov/nuccore/) to produce a phylogenetic tree of the genus. Based on a previous phylogeny of *Dioscorea*, the species *D. bulbifera* was selected as the outgroup because of its distance from the section *Stenophora* to which the North American species belong (Al-Shehbaz & Schubert 1989; Raz 2002; Wilkin et al. 2005). This was done for the resulting sequences from *matK*, *trnH-psbA*, *ccsA-ndhD*, and *rrn4-5-trnN*. Each tree was analyzed heuristically in WinClada (500 replicates, holding 2 trees in each replicate) and through a bootstrap analysis of 500 replications. The resulting bootstrap values are included at each of the well-supported branches of each phylogeny. These are the numbers seen before the branch point in each of the following phylogenies.

When analyzed, the set of data from *matK* sequences from the genus *Dioscorea* resulted in four most parsimonious trees. The length was 83. The consistency index (CI) equaled 83 while the retention index (RI) equaled 97. The character states are shown on phylogeny as indicated by the solid and open circles on the phylogeny. The branch on which the samples from this study—*D. villosa* (306), *D. villosa* (307), and *D. quaternata* (308)—can be found contains specimens from section *Stenophora*. A phylogeny was also generated for the plastid *trnH-psbA* which can be seen below (Figure 5).

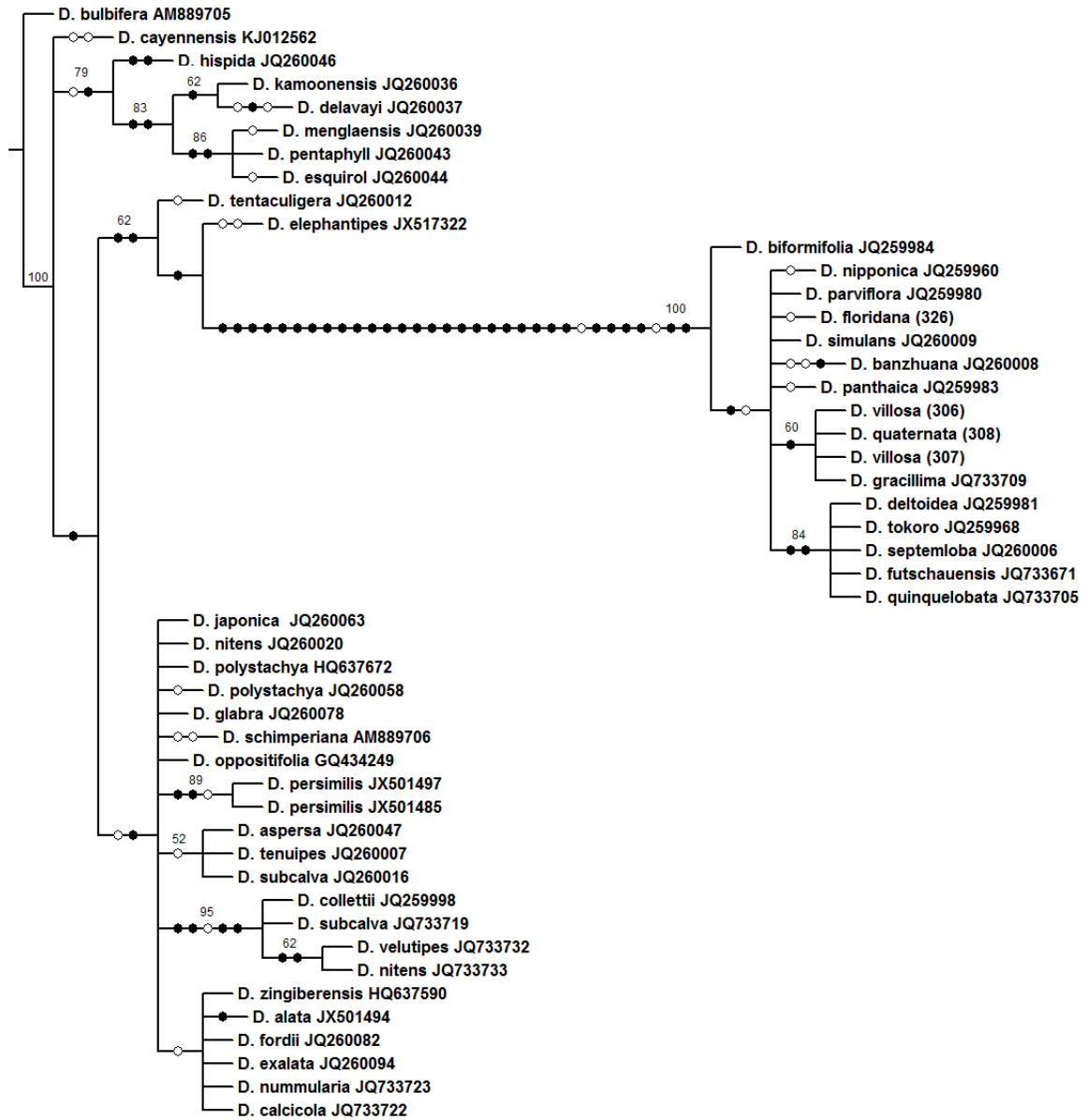


Figure 4. The most parsimonious tree of *Dioscorea* based on plastid *matK* DNA data. Dots represent character state changes. Numbers above branches indicate bootstrap support values. Samples with letter-number combinations (GenBank accession numbers) following the name were downloaded from GenBank.

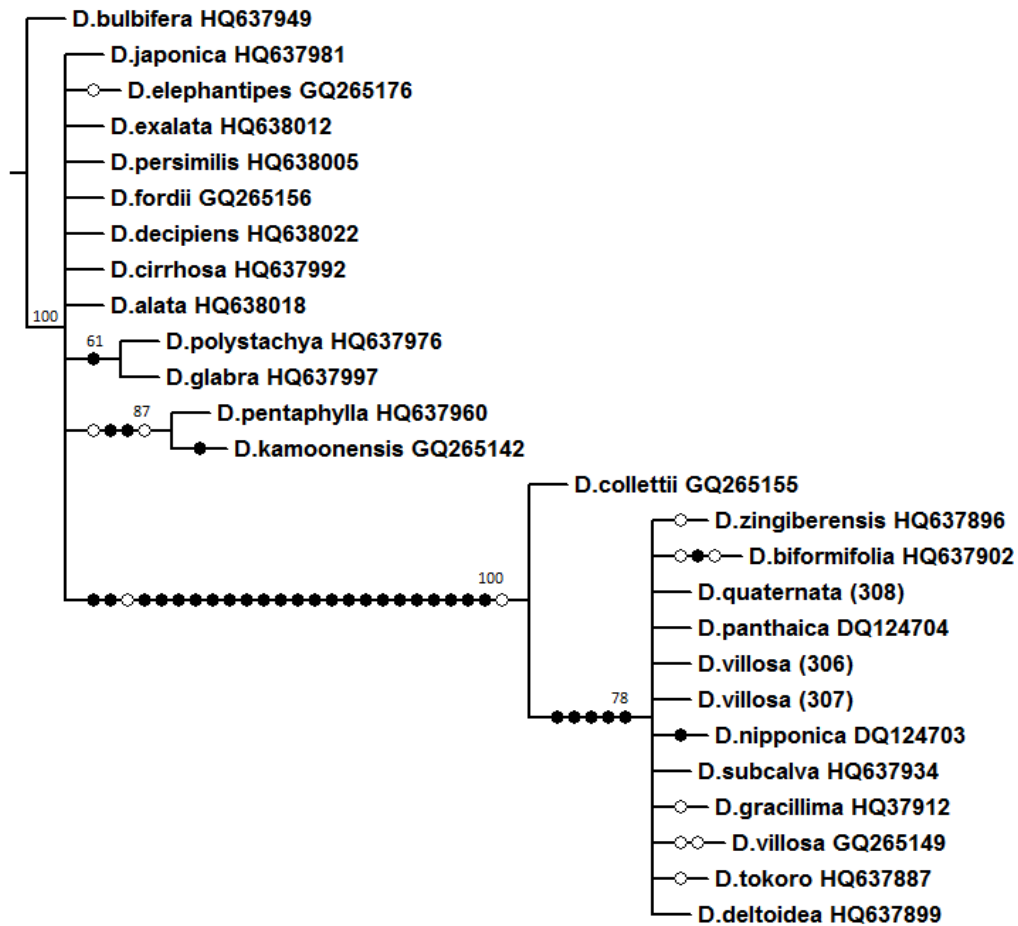


Figure 5. The most parsimonious tree of *Dioscorea* based on plastid *trnH-psbA* DNA data.

The analysis of these data resulted in four most parsimonious trees with a length of 44, CI of 86, and RI of 98. The unresolved branch with *D. villosa* (306), *D. villosa* (307), *D. quaternata* (308), and *D. floridanta* (326) represents sect. *Stenophora*. The DNA regions from *ccaA-ndhD*, *rrn4-5-trnN*, and *rpl32-ndhF* could not be analyzed with as numerous of a collection of other species based on the fact that fewer sequences of these DNA regions have been posted on GenBank. The analysis of the DNA region of *ccaA-ndhD* can be seen in Figure 6.

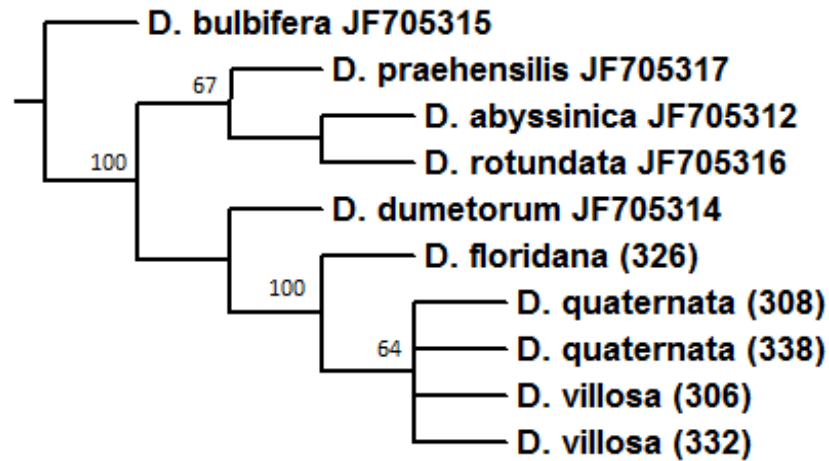


Figure 6. The most parsimonious tree of *Dioscorea* based on plastid *ccsA-ndhD* DNA data.

When the analysis was run, one tree was recovered. The length was 412. The CI and RI both equaled 99. Characters were not marked on this tree because of the large distance between the North American species and *D. dumetorum*. The distance was a total of 382 characters. Next, a phylogeny was generated from plastid *rrn4-5-trnN*.

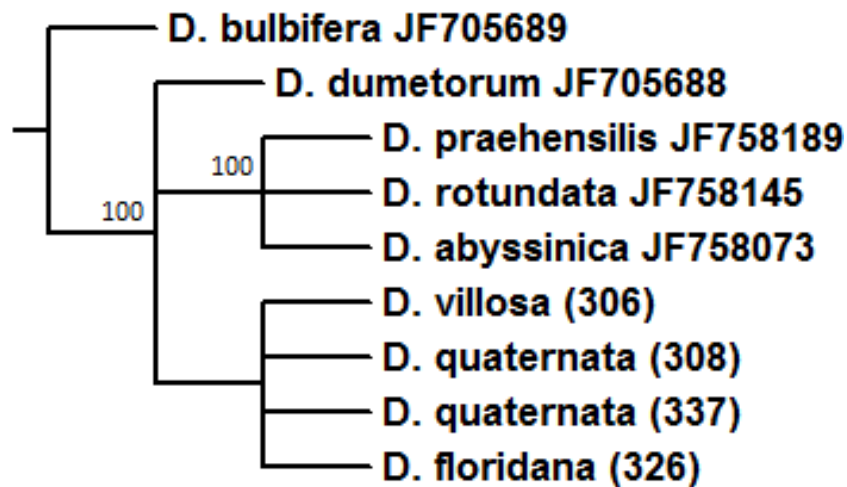


Figure 7. The most parsimonious tree of *Dioscorea* based on plastid *rrn4-5-trnN* DNA data.

These data resulted in two most parsimonious trees, with a length of 228. Again, the CI and the RI both equaled 99. The characters of this analysis were also not marked because of the large distance between the North American species and the remaining species. The distance was a 225 character difference. A tree was not constructed for *rpl32-ndhF* due to the fact that an insufficient (less than five) number of DNA sequences were available on GenBank for comparison.

A tree was also generated from the *matK* data focusing on species from the section *Stenophora* in hopes of better resolution of the closest related species. DNA sequences were pulled from the same sampling used in Figure 4. Several outgroups were included from other sections of the *Dioscorea* genus.

These data resulted in one tree, with a length of 68. The CI equaled 91, while the RI equaled 97. The tree contains seven species outside of the section *Stenophora*: *D. bulbifera*, *D. delvayi*, *D. subclava*, *D. persimilis*, *D. zingiberensis*, *D. nummularia*, and *D. elephantipes*. The remaining species belong to sect. *Stenophora*.

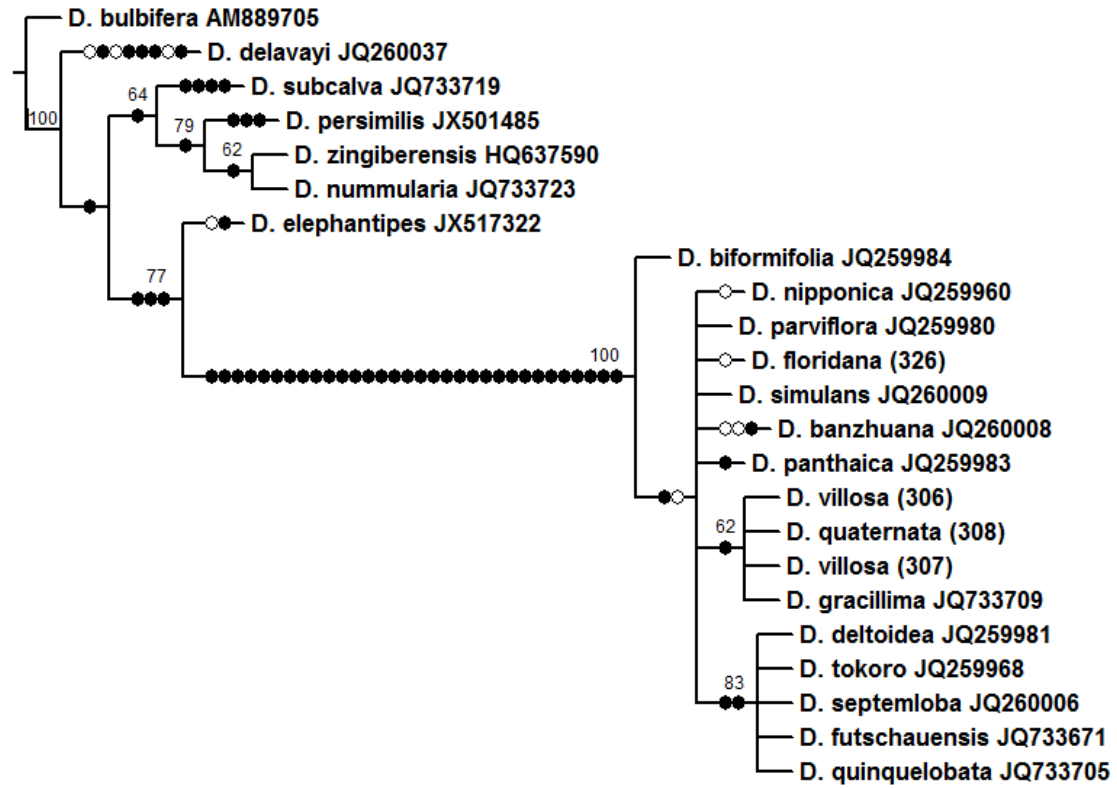


Figure 8. The most parsimonious tree of *Dioscorea* sect. *Stenophora* based on plastid *matK* DNA data.

CHAPTER V

DISCUSSION

The results did not affirm the hypothesis that the species complex of *D. villosa* consists of several species. The variation seen between *D. floridana* and the putative species was as expected. These variations indicated that *D. floridana* is a distinct species, which matches the consensus of the literature. Diagnostic variation was also expected among the putative species; however, the resulting lack of genetic variation in four of the five DNA regions among the morphologically different putative species is mostly ambiguous about species boundaries within the *D. villosa* complex. This lack of variation indicates that the complex is (1) actually just a single species of morphologically variable individuals OR that (2) variable DNA regions are yet to be found which support the hypothesis of differentiation. However, the variation observed in the DNA region *rpl32*—*ndhF* counters the hypothesis. Similarity in the sequences was observed across the various putative species, meaning some variation grouped specimens of *D. villosa* with *D. quaternata* while others grouped another specimen of *D. villosa* with another specimen altogether. In other words, the aligned points of various species show variation in groups, but they do not match with the morphological groupings. These odd pairings imply that these variations are the result of *intraspecific* variation as opposed to distinguishing characters. This explanation is supported by the fact that *rpl32*—*ndhF* has been utilized in studies for population genetics, which utilizes *intraspecific* variation to study population dynamics (Scarelli et al. 2011). This also could have been seen in the

rrn4-5-trnN and *ccsA-ndhD*, which have also been used for population genetics in monocotyledons (Scarelli et al. 2011). These results indicate that these two regions are not effective for population genetic studies of *D. villosa*. The lack of variation in the gene sequences does not match the variation seen in the morphological features which indicates the presence of gene flow among the putative units or retention of ancestral polymorphism. Therefore, the observations indicate a lack of divergence in the evolution of the specimens studied. Overall, the observations do indicate that *D. villosa* is likely one species which consists of varying traits implying that the morphological differences do not represent the character states described by Nixon and Wheeler (1990). These results support the conclusions of Raz (2002), Britton and Brown (1970), Weakley (2012), and similar floras. These findings also call into question the hypothesis of many like Al-Shehbaz & Schubert, Gleason, Clewell, Ward, Small, and Deam (1989; 1991; 1985; 1977; 1933; 1940). With this conclusion in mind, the maps compiled from the readings were revisited and adjusted into one which reflects the findings (Figure 9).

While the conclusions from these data indicate that the species in question does not require subdivision, it is difficult to say that the issue will never need to be revisited. There have always been difficulties determining variation in plants due to the lack of variation seen in many plastid regions. The absence of variation at one particular locus or a set of loci does not absolutely contradict a hypothesis of lineage separation; it could simply mean that the species in question may still be in the early stages of divergence resulting in fewer points of variation for sampling (de Queiroz 2007). Though many

regions were tested, there could still be a number of plastid or nuclear regions which could produce the expected variation patterns.

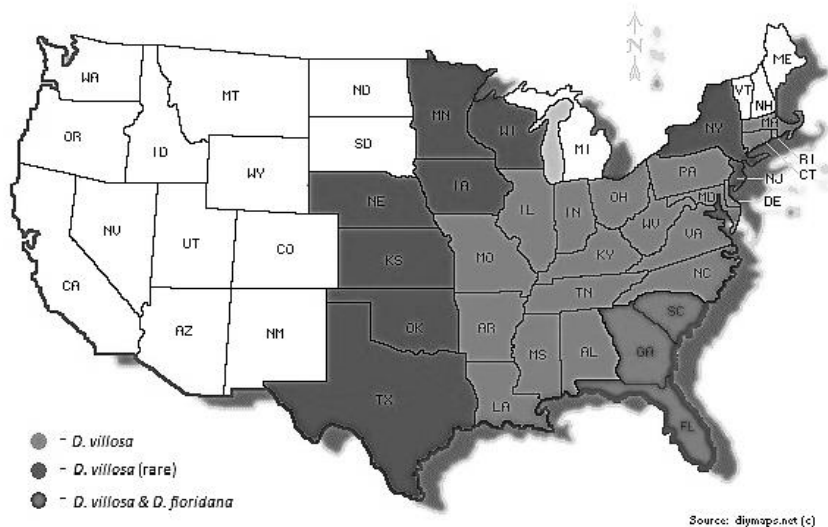


Figure 9. Distribution of *Dioscorea villosa* and *D. floridana*, based on national and regional treatments of the genus.

In addition to low level or lack of variation, issues arose when obtaining sequences. The fact remains that of 11 primer regions only 5 were successfully sequenced. Even with the addition of special mixes designed to increase chances, many attempts never bore fruit. This is likely the result of contaminants (Olmstead & Palmer 1990). Olmstead and Palmer suggest irradiating the PCR mixture for 3 minutes prior to the addition of the sample DNA as a possible solution (1990). This would not always be effective. Some sequences like the ITS region did result in amplified DNA regions, but the resulting sequences turned out to be fungal containments that likely originated in the *Dioscorea* samples themselves, as *Dioscorea* have endophytic fungi (Xu et al. 2008). The tag-along contaminant is more readily amplified making it nearly impossible to get a

sequence from the sample of interest. This issue could be subverted according to White et al. (1990) who suggest utilizing the fungus DNA for a phylogenetic study based on the idea that communalistic partners evolve in similar patterns, meaning a study of the variation in the fungal sequences from each of the putative species could potentially give insight into their own relationships. Therefore, these conclusions might warrant further examination.

The data that were obtained were also utilized in conjunction with similar sequences from other species from the genus *Dioscorea* to complete phylogenies for comparison with the pre-existing phylogeny. These phylogenies did not include samples of the native North American species of *Dioscorea*. Since the available number of samples was fairly small for most of the phylogenies generated, resolution of the relationships was difficult. The relationships that were shown are likely accurate based on the high values of CI and RI, in addition to the high bootstrap values seen at the branching points. Though not highly resolved and not very precise given the sampling, the groups recovered are congruent with the groups obtained in the plastid gene phylogeny constructed by Wilkin and colleagues (2005). The Wilkin et al. (2005) phylogeny did not contain all of the same species, but closely related species to the ones sampled. In the case of *matK* and *trnH-psbA*, the placement of the native North American species was as expected, related to species from section *Stenophora* (Gao et al. 2008). In the case of *ccsA-ndhD* and *rrn4-5-trnN*, no representative species of *Stenophora* were obtained; however, the large distance recovered between the native North American species and the other samples indicates that they do, in fact, belong with

the section *Stenophora*. There is a great deal of divergence between the section *Stenophora* and other section of yams as a result of this being the only section of yams that has a rhizome as opposed to a tuber or something equivocal (Wilkin et al. 2005). These past findings in addition to the results of this paper further support that *Stenophora* is a monophyletic group. This paper supports the fact that the native North American species belong to this group.

Further analysis of the North American species in relation to other species of section *Stenophora* was produced in an attempt to better understanding of their relationships to the genus as a whole. The phylogeny utilized the *matK* plastid region based on previous success (Gao et al. 2008). The resulting phylogeny did not result in optimal resolution, but it did compliment the expected relationship determined by past studies at the best points of resolution. For example, *D. biformifolia* was the least related to all other specimens in both phylogenies (Gao et al. 2008). While Figure 8 does not clarify the closest relations of *D. floridana*, it does indicate that *D. villosa* is related to *D. gracillima*, a Japanese species. This grouping is supported by a solid character and a strong bootstrap number which indicated a supported branch. Further attempts to clarify the relationships of the North American species within *Dioscorea* sect. *Stenophora* could utilize the nuclear region *pgi* based on alternate studies of the section (Kawabe et al. 1997).

CHAPTER VI

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